

Visualizing the dynamics of viral replication in living cells via Tat peptide delivery of nuclease-resistant molecular beacons

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In this study, we describe the use of nuclease-resistant molecular beacons (MBs) for the real-time detection of coxsackievirus B6 replication in living Buffalo green monkey kidney (BGMK) cells via Tat peptide delivery. A nuclease-resistant MB containing 2'-O-methyl RNA bases with phosphorothioate internucleotide linkages was designed to specifically target an 18-bp 5' noncoding region of the viral genome. For intracellular delivery, a cell-penetrating Tat peptide was conjugated to the MB by using a thiol-maleimide linkage. Presence of the Tat peptide enabled nearly 100% intracellular delivery within 15 min. When the conjugate was introduced into BGMK cell monolayers infected with coxsackievirus B6, a discernible fluorescence was observed at 30 min after infection, and as few as 1 infectious viral particle could be detected within 2 h. The stability and the intracellular delivery properties of the modified MBs enabled real-time monitoring of the cell-to-cell spreading of viral infection. These results suggest that the Tat-modified, nuclease-resistant MBs may be powerful tools for improving our understanding of the dynamic behavior of viral replication and for therapeutic studies of antiviral treatments.

detection | infectious diseases | real time

Real-time detection of mRNA in living cells is essential to provide information on the dynamics of RNA expression and localization and can be an important tool for advancement in disease pathophysiology, drug discovery, and medical diagnostics. In particular, the ability to monitor the real-time replication of viruses in living cells is vital for the rapid detection of viral infection and an understanding of viral pathogenesis.

Among the technologies currently under development for gene detection in living cells, perhaps the most promising one is molecular beacons (MBs). MBs are single-stranded oligonucleotide probes possessing a stem-loop structure; they are double labeled with a fluorophore at one end and a quencher at the other (1). These probes are specific for a target nucleotide sequence and produce fluorescence upon target binding. The spontaneous hybridization between MBs and their target sequences is highly specific and can even distinguish a single nucleotide mismatch (2–4). Recently, MBs have been used to detect the presence of viral RNA in infected cells, with positive responses to even a single infectious viral particle (5). However, the long-term, real-time monitoring of viral RNA in living cells has not been possible because of the relatively short half-life (≈ 30 min) of MBs due to endogenous nuclease degradation, resulting in false-positive signals (6, 7). It has been shown that modifications of MBs with 2'-O-methyl RNA bases and phosphorothioate internucleotide linkages can be used to significantly improve duplex stability and nuclease resistance (8–13). It is easy to envision that such an MB design can be adapted for the real-time monitoring of viral infection.

In addition, real-time monitoring of viral replication using MBs has been hindered by the lack of an efficient and noninvasive method for intracellular delivery. Conventional approaches such as transfection are slow and inefficient. Delivery based on streptolysin O is faster (≈ 2 h) but can be used only in ex vivo cellular assays. Even microinjection is not suitable for viral detection, because one

cannot predict a priori which cells are infected by viruses. Among the many noninvasive intracellular delivery methods, the HIV-1-derived Tat peptide-based method is the most efficient and has been shown to retain the ability to intracellularly deliver various conjugated cargoes (14–16). This Tat-based method, when combined with nuclease-resistant MBs, could provide a powerful means for rapid detection and real-time monitoring of viral replication in living cells with high specificity and sensitivity.

By delivering nuclease-resistant MBs into host cells before viral infection and subsequently tracking fluorescent hybrids with viral RNA, we envision that a better understanding of the dynamic behavior of viral replication could be gained through in vivo experiments and could extend to therapeutic studies of antiviral treatments. Herein, we report the utilization of a nuclease-resistant MB conjugated with a Tat peptide to examine the coxsackievirus B6 (CVB6) viral reproductive cycle in living host cells.

Results and Discussion

Design and Characterization of Nuclease-Resistant MB. An MB (MB CVB6-Tat) targeting an 18-bp region of the 5' untranslated region of CVB6 was designed. The DNA backbone was modified with sulfur-substituted 2'-O-methyl oligoribonucleotides for improved nuclease resistance (17–20). A Tat peptide for intracellular delivery was conjugated to the thiol group at the quencher end by using a maleimide group placed at the N terminus of the peptide (Fig. 1A) (21, 22). As expected, the modified MBs were highly resistant to nuclease cleavage by DNase I (Fig. 1B). In contrast, an unmodified MB was susceptible to nuclease degradation, resulting in almost instantaneous increase in fluorescence. The dual modifications had no effect on the hybridization kinetics of the MB, as a rapid increase in fluorescence was observed in the presence of a complementary target (Fig. 1C).

Intracellular Delivery of Tat-Modified MB CVB6. The intracellular delivery efficiency was tested by incubating 0.5, 1, or 2 μ M MB–target hybrids with a monolayer of Buffalo green monkey kidney (BGMK) cells. Fluorescence was detectable in 100% of the living cells as early as 15 min after introduction of the MB–target hybrids (Fig. 2A). The time-lapse images showed that the cellular uptake increased after 15 min and reached saturation after 1 h of incubation. The fluorescence intensity was constant for up to 12 h, indicating that the MB–target hybrids were retained inside the cells after delivery and remained resistant to the intracellular RNase H.

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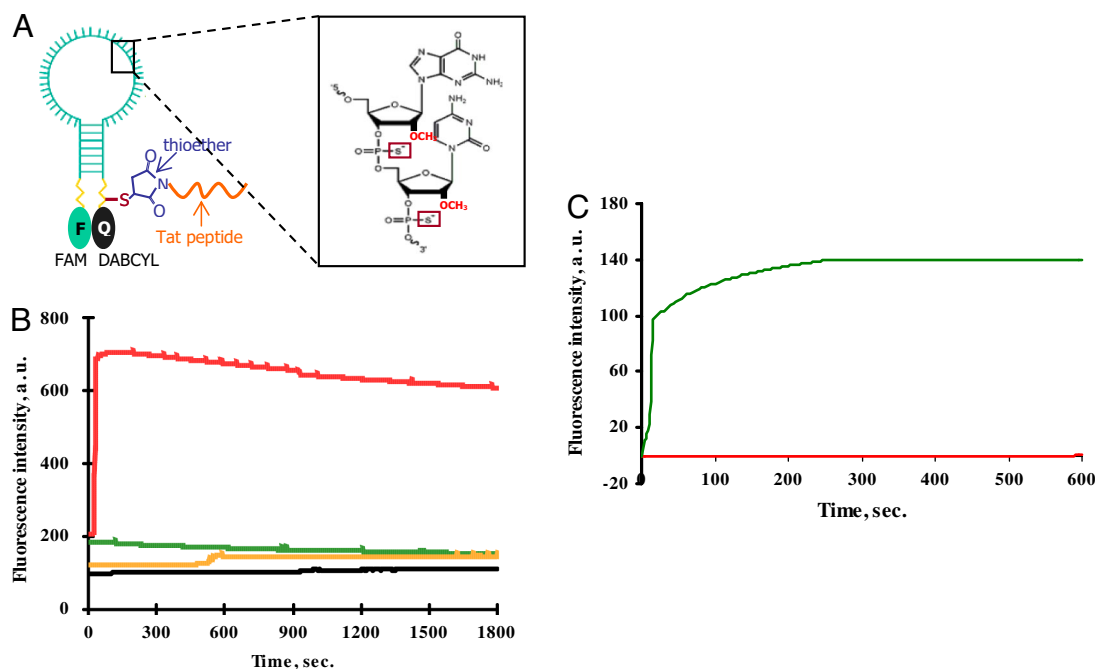


Fig. 1. MB backbone modification and nuclease sensitivity study. (A) A schematic representation of the Tat-modified, nuclease-resistant MB. The phosphodiester bond was modified by replacing a nonbridging oxygen with sulfur and the 2'-sugar deoxy with 2'-O-methyl group. At room temperature, the thiol group at the quencher end reacted (≈ 2 h) with a maleimide group placed at the N terminus of the peptide to yield a chemically stable thioether bond. (B) Nuclease sensitivity assays using ribonuclease-free DNase I. The fluorescence of the nuclease-resistant MB is shown in yellow, and the fluorescence of an unmodified MB is shown in red. The background fluorescent signals (shown in black and green) without DNase I addition are also shown. (C) Kinetics of hybridization of Tat-modified MB CVB6 with (green) or without (orange) complementary oligonucleotides.

Unhybridized MB was also introduced into BGMK cells, and no fluorescence was detected during the same 12-h period, again indicating the intracellular resistance of the modified MBs to nuclease attack (Fig. 2B). In contrast, in the absence of Tat, no internalization of MBs was observed, and fluorescence was detected only in the medium (Fig. 2B), confirming the effectiveness of the Tat peptide for rapid intracellular delivery.

Detection of CVB6 Infection by Tat-Modified MBs. After validating the properties of Tat-modified MBs, their ability to detect viral RNA was tested. A confluent monolayer of BGMK cells was first incubated with $1 \mu\text{M}$ MB for 30 min before being infected with 10-fold serial dilutions of CVB6, followed by fluorescence microscopy. Compared with uninfected cultures (0 pfu), where no fluo-

rescent cells were present independent of time, a greater number of fluorescent cells were detected at 2 h post infection (p.i.) for the culture infected with a viral dosage corresponding to 1 pfu (Fig. 3A). The higher number of fluorescent cells compared with plaque-forming units is likely due to the fact that not all viruses that infect a cell are necessarily able to complete the replication cycle (23). This result is also consistent with the higher infectious virus titers observed by using the quantal assay, which is based on the direct microscopic viewing of cells for virus-induced cytopathic effects, rather than the plaque assay (24). This 2-h detection window is substantially faster than a similar approach reported using cell fixation/permeabilization (16). It is possible that the rapid and noninvasive intracellular delivery enables hybridization with viral RNA to occur shortly after virus uncoating without the possibility

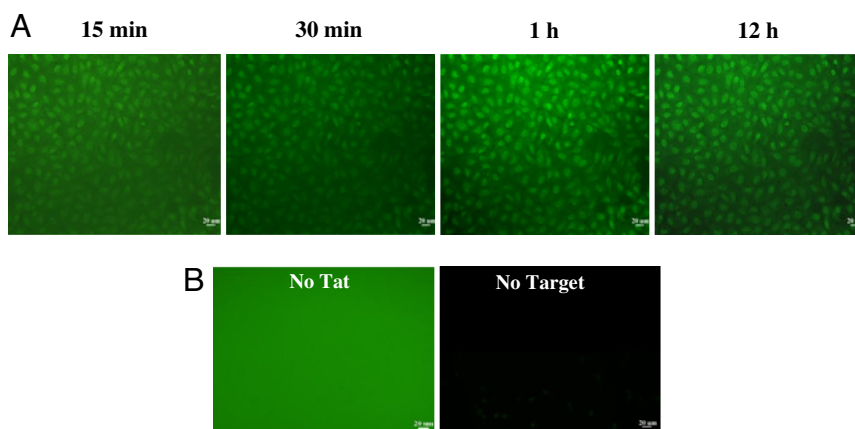


Fig. 2. Intracellular delivery of MB CVB6-Tat-target hybrids (A) or MB without Tat modification or without targets (B). BGMK cells were incubated with $1 \mu\text{M}$ MB for 12 h, and images were captured by using a fluorescent microscope. (Scale bar, $20 \mu\text{m}$.)

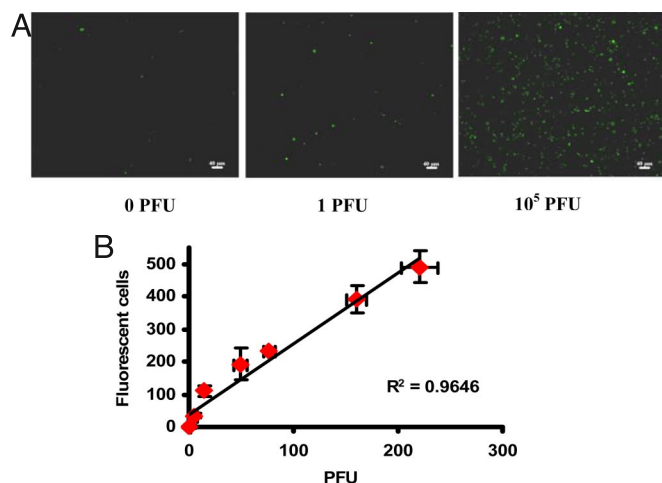


Fig. 3. In vivo detection of CVB6 in BGGMK cells. (A) Visualization of BGGMK cells infected with 0, 1, or 10^5 pfu at 2 h p.i. (B) The correlation between the number of plaque-forming units and fluorescent cells at 2 h p.i. Error bars represent the standard deviation of 3 replicate experiments. (Scale bar, 40 μ m.)

of degradation caused by fixation/permeabilization. To our knowledge, detection of 1 pfu of CVB6, or any other enterovirus, at 2 h p.i. has never been reported.

Using the 2-h infection window, we tested the utility of Tat-modified MBs to quantify infectious CVB6 dosages. Cells were infected with 1–200 pfu of CVB6 per well, and the average number of fluorescent cells was recorded. A linear correlation was obtained by plotting the number of fluorescent cells versus plaque-forming units (Fig. 3B). The number of plaque-forming units can be determined easily by using the correlation obtained after direct counting of fluorescent cells. More importantly, the method can be used to provide rapid quantification of infectious CVB6 dosages within 2 h p.i. compared with the minimum 48-h incubation period for the plaque assay.

Real-Time Monitoring of Cell-to-Cell Spreading of CVB6. The ability to detect infected cells continuously should allow one to follow the spreading of infectious viruses on a real-time basis. To determine whether this was indeed possible, BGGMK cells were infected at a very low infection dosage (multiplicity of infection: 0.01 pfu/cell) and monitored continuously by using a fluorescence microscope in

a fixed area for 12 h. Fig. 4 shows the cell-to-cell progression of virus spreading at 6 representative time points [A real-time movie of virus spreading is provided in supporting information (SI) [Movie S1](#)]. Several infected cells were observed at 15 min p.i., suggesting that the viruses entered the cells and started the uncoating process within 15 min. The number of fluorescent cells slowly increased with time, suggesting continuous virus infection. By 6 h p.i., a further outward spread of fluorescent cells was observed, indicating the secondary spreading of infection from progeny virions to cells surrounding the initial infected cells. The number of fluorescent cells continued to increase with time, and infection spread outward to the entire observation area by 12 h p.i. The majority of infected cells remained adherent, and some fluorescence was visible outside the cells, indicating that the fluorescent hybrids with viral RNA entered the extracellular region as a result of the release of progeny virions during cell lysis.

In summary, we demonstrated the use of nuclease-resistant, Tat-modified MB for real-time monitoring of viral replication and infection. This method is simple because it requires no cell pre-treatment (e.g., fixation/permeabilization) and can be used to study cell-to-cell viral spreading. This method is particularly attractive when applied to viruses with very slow growth, nonlytic viruses, and those that do not produce detectable cytopathic effects in infected cells. Compared with conventional viral plaque assays, which detect infection based on cell lysis only and may take days to weeks to complete, this real-time approach provides an opportunity to study the progress of the entire infectious cycle.

Materials and Methods

BGMK Cell Culture. BGMK cells obtained from American Type Culture Collection (passages 50–60) were grown in 400 mL of $1\times$ autoclavable minimum essential medium (AMEM; Irvine Scientific) containing 4 mL of 7.5% NaHCO_3 , 8 mL of 1 M HEPES, 4 mL of nonessential amino acids (NEAA; Gibco-BRL), 10 mL of A/B-L (1,000 units/mL penicillin, 1,000 units/mL streptomycin, 2 mg/mL kanamycin, 2,000 units/mL nystatin, 80 mM L-glutamine), and 40 mL of FBS (Sigma-Aldrich) at 37°C in a 5% CO_2 atmosphere. PBS [$1\times$ PBS = 0.01 M phosphate (pH 7.4), 0.138 M NaCl, and 2.7 mM KCl] and Tris-buffered saline solution [$1\times$ TBSS = 0.05 M Tris (pH 7.4), 0.28 M NaCl, 10 mM KCl, and 0.82 mM Na_2HPO_4] were used for washing steps in the plaque assay and MB analysis, respectively.

Virus Preparation. Virus stocks of CVB6 Schmitt strain (ATCC VR-155) were allowed to proliferate on BGGMK cells for 2 days at 37°C in a 5% CO_2 atmosphere and collected by freeze-thawing (3 times) infected flasks demonstrating >80% lysis and extracting the cell lysate with chloroform. The CVB6 virus stock was stored at -80°C .

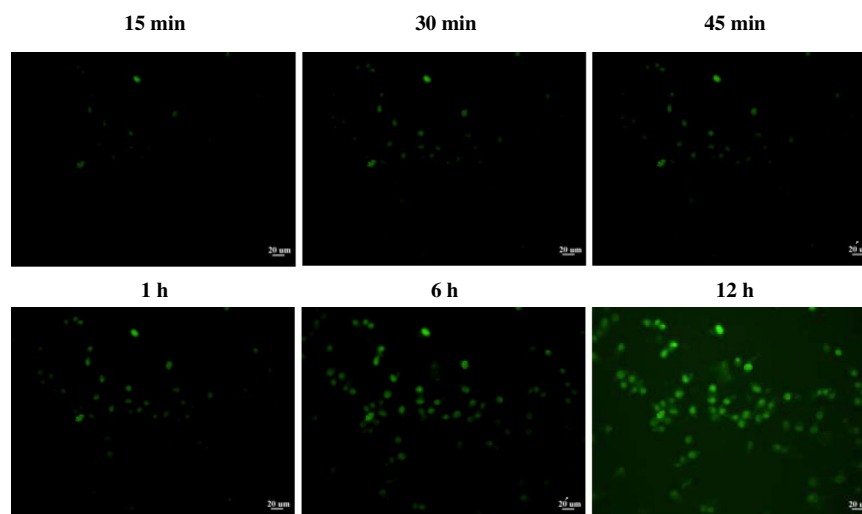


Fig. 4. Real-time detection of viral spreading. BGGMK cells were first incubated with 1 μ M MB, infected with CVB6 at an multiplicity of infection of 0.01 pfu/cell, and monitored by using a fluorescent microscope. (Scale bar, 20 μ m.)

Plaque Assay. The CVB6 virus stock was thawed, and then a series of 10-fold serial dilutions in 1× PBS were prepared. Confluent, 1-day-old BGMK cell monolayers in 12-well, 22.1-mm dishes (Costar; Corning) were infected with 1 mL of virus dilution. After 90 min of adsorption at room temperature, the solutions were aspirated, and 1 mL of 100 mL of 2% carboxymethylcellulose (CMC) sodium salt (Sigma–Aldrich) containing 100 mL of 2× AMEM (Irvine Scientific) with 2 mL of 7.5% NaHCO₃, 4 mL of 1M HEPES, 2 mL of NEAA, 5 mL of A/B-L, and 4 mL of FBS (Sigma–Aldrich) was added to each well. After 2 days of incubation at room temperature, the CMC overlay was removed, and the cells were treated with 0.8% crystal violet/3.7% formaldehyde solution overnight. Excess stain was removed by washing with de-ionized water and the virus plaques were counted.

Design of Nuclease-Resistant MB. MB CVB6 was designed on the basis of an alignment of the sequences of enterovirus strains obtained from GenBank database. The DNA folding program *mfold* (www.bioinfo.rpi.edu/) and *IDT SciTools* (www.idtdna.com/SciTools/SciTools.aspx) were used to predict the thermodynamic properties and the secondary structures of MBs. MB CVB6 5'-6-FAM (fluorescein)-GCCGCTCGCATTAGGGGCCGAGAGC/thiol-dG/GC-DABCYL-3' [stem sequence is underlined; DABCYL is 4-(4-dimethylaminophenylazo)benzoic acid] possessing a 2'-O-methylribonucleotide backbone with phosphorothioate inter-nucleotide linkages was synthesized (TIB Molbiol) to be specifically hybridized to an 18-bp region of the 5' untranslated region of the enterovirus genome. The thiol group on the quencher end is for the reaction with a maleimide group attached to the N terminus of the Tat peptide to form a thiol–maleimide bridge. MB CVB6 was suspended in 100 mM Tris-HCl (pH 8.0) buffer containing 1 mM MgCl₂ to make the concentration 100 μM for the subsequent studies.

DNase Sensitivity. To test the nuclease sensitivity of MB CVB6, the fluorescence of a 500-μl solution of 1 μM MB CVB6 was recorded as a function of time at room temperature. Five units of ribonuclease-free DNase I was added, and the fluorescent signals (excitation 495 nm, emission 521 nm) were measured for 30 min by using an RF-551 spectrofluorometric detector (Shimadzu). As a comparison, the fluorescence of a 500-μl solution of 1 μM MB possessing deoxyribonucleotide backbones 5'-6-FAM-GCCGCTCGCATTAGGGGCCGAGAGCGCGC-DABCYL-3' (MB CVB1, stem sequence is underlined) was recorded after adding 5 units of ribonuclease-free DNase I. Without adding ribonuclease-free DNase I, the background fluorescent signals of a 500-μl solution containing 1 μM MB CVB6 or MB CVB1 were monitored for 30 min.

Peptide Conjugation. One hundred and fifty micromolar N-terminal maleimide-modified Tat peptide H-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-N-CH₂CH₂-N-maleimide (Global Peptide) was mixed with 100 μM thiolated MB in the dark for 2 h to form a stable thiol–maleimide linkage. The peptide-linked MB complex was dialyzed overnight in Slide-A-Lyzer Mini Dialysis Units, 10,000 molecular weight cutoff, to remove the unconjugated peptide/MBs (Pierce). The peptide-conjugated MB CVB6-Tat was stored at –20°C until used for experiments.

Cellular Delivery of Peptide-Conjugated MBs. BGMK cells were seeded into the 8-well Lab-Tek Chambered Coverglass (Fisher Scientific) at 37°C in 5% CO₂ in air

and cultured to >90% confluence. After removal of the incubation medium, the cell monolayer was washed twice with 1× TBSS. To facilitate determining the efficiency of Tat peptide-mediated intracellular delivery, nonconjugated MB CVB6 or MB CVB6-Tat was mixed with complementary oligonucleotides (5'-CTCCGGCCCCCTGAATGCG-3') to the loop region at an MB/oligonucleotide molar ratio of 1:1. BGMK cells were incubated at 37°C in the dark with 1× Leibovitz L-15 medium (Invitrogen) containing either preformed nonconjugated MB CVB6 hybrids or MB CVB6-Tat hybrids at MB concentrations of 0.5, 1, or 2 μM. The Leibovitz L-15 contains no phenol red to eliminate autofluorescence and to increase optical transmission. To record the image, the chamber well was placed on the Zeiss Axiovert 40 CFL inverted fluorescence microscope stage and was marked to permit the repeated observation of the chosen region in the cell monolayer. As soon as the positive fluorescent signals were observed inside the cells, the chamber well was kept on the microscope stage instead of returning it to the 37°C incubator. All assays were carried out over a period of 12 h, and the fluorescence images were taken at intervals of 5 min.

Progression of Viral Infection in Living Cells. BGMK cells were cultured to >90% confluence in the 8-well Lab-Tek Chambered Coverglass (Fisher Scientific) at 37°C in 5% CO₂ atmosphere. After incubation for predetermined time periods, the slides were removed from the 37°C incubator, and the growth medium was aspirated. Following 2 washes with 1× TBSS, the cells were incubated with 1 μM MB CVB6-Tat in 1× Leibovitz L-15 medium (Invitrogen) at 37°C in the dark for 30 min. Without washing away the incubation medium, the chamber wells were oriented on the microscope stage; the cells were infected with 10-fold virus dilutions in 1× Leibovitz L-15 medium and were observed under the Zeiss Axiovert 40 CFL inverted fluorescence microscope at room temperature for 12 h. The fluorescence images were recorded at intervals of 5 min.

Fluorescence Microscopy and Image Processing. Living cell imaging was performed on a Zeiss Axiovert 40 CFL inverted microscope equipped with a 12-V, 35-W halogen lamp (for the phase-contrast images) and an HBO 50 W/AC mercury lamp (for the fluorescence images). The objectives used were a 5×/0.12 A-Plan, a 10×/0.25 A-Plan, a 20×/0.50 EC Plan-NEOFLUAR, and a 40×/0.50 LD A-Plan (Zeiss). 6-FAM-labeled fluorescent hybrids were detected by using a filter set consisting of a D480/30-nm exciter, a D535/40-nm emitter, and a 505-dichroic long pass beam splitter (Chroma Technology). Images were acquired by using a ProgRes MF^{scan} Monochrome CCD camera (Jenoptik). Both phase-contrast and fluorescence images were analyzed by using Image-Pro PLUS analysis software (Media Cybernetics). All settings for image processing were kept constant, and the exposure time for image capture was adjusted, if necessary, to maintain output levels similar to those observed under the fluorescence microscope.

Enumeration of Fluorescent Cells. To calculate the infected cells (fluorescent cells) in each chamber well, 30 fields within the well were randomly chosen, and the fluorescence images were collected at 10× magnification. The number of fluorescent cells within the area was counted by Image-Pro PLUS analysis software.

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